Journal of Experimental Botany, Vol. 62, No. 6, pp. 2023–2038, 2011
doi:10.1093/jxb/erq393 Advance Access publication 3 January, 2011

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Functional characterization of the three genes encoding 1-deoxy-D-xylulose 5-phosphate synthase in maize

Elizabeth Cordoba[1,*], Helena Porta[1,*], Analilia Arroyo[1,†], Carolina San Román[1], Luis Medina[1], Manuel Rodríguez-Concepción[2] and Patricia León[1,‡]

[1] Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001 Chamilpa. Apdo. Postal 510-3 Cuernavaca, Morelos 62210, Mexico
[2] Department of Molecular Genetics, Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB, 08034 Barcelona, Spain

* These authors contributed equally to this work.
† Present address: Centro de Investigación en Biotecnología Aplicada, Instituto Politécnico Nacional, Ex-Hacienda San Juan Molino Carrretera estatal Tecuexcomac Tepetitla Km. 1.5 Lardizabal, Tlaxcala, CP 90700, México.
‡ To whom correspondence should be addressed. E-mail: patricia@ibt.unam.mx

Received 8 September 2010; Revised 9 November 2010; Accepted 15 November 2010

Abstract

The 1-deoxy-D-xylulose 5-phosphate synthase (DXS) enzyme catalyses the first biosynthetic step of the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. In plants the MEP pathway is involved in the synthesis of the common precursors to the plastidic isoprenoids, isopentenyl diphosphate and dimethylallyl diphosphate, in plastids. DXS is recognized as limiting this pathway and is a potential target for manipulation to increase various isoprenoids such as carotenoids. In *Zea mays* three *dxs* genes exist that encode plastid-targeted functional enzymes. Evidence is provided that these genes represent phylogenetically distinctive clades conserved among plants preceding monocot–dicot divergence. There is differential accumulation for each *dxs* gene transcript, during development and in response to external signals such as light. At the protein level, the analysis demonstrates that in *Z. mays,* DXS protein is feedback regulated in response to the inhibition of the pathway flow. The results support that the multilevel regulation of DXS activity is conserved in evolution.

Key words: 1-Deoxy-D-xylulose 5-phosphate synthase (DXS), isoprenoid biosynthesis, maize, 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, post-transcriptional regulation.

Introduction

Plants produce many isoprenoids that are functionally important in essential processes. Despite their diversity, all isoprenoids are formed from two common precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Additionally isoprenoids are of biotechnological, medical, and industrial importance (Lange and Croteau, 1999; Römer *et al.*, 2000, and references within), prompting efforts to increase levels of these valuable compounds.

In plants, the biosynthesis of IPP and DMAPP utilizes two independent pathways that have different precursors. The acetate/mevalonate (MVA) pathway is found in most eukaryotes. The second pathway known as the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway operates in the chloroplast of photosynthetic eukaryotes and also in most eubacteria and in apicomplexa parasites (Eisenreich *et al.*, 2004). Thus, the genes and enzymes of the MEP pathway are attractive targets to develop new antibacterial and antiparasitic drugs, and herbicides (Rodríguez-Concepción, 2004; Rohdich *et al.*, 2005). Although evidence supports a limited exchange of intermediates between these two pathways, it is clear that only one pathway is primarily responsible for the synthesis of particular isoprenoids. The MVA pathway provides the precursors for sesquiterpenes (C_{15}) and triterpenes (C_{30}) (Lichtenthaler, 1999; Eisenreich *et al.*, 2001). In contrast,

the MEP pathway supports the synthesis of the major photosynthetic pigments (chlorophylls and carotenoids), hormones (gibberellins and abscisic acid), and mono- and diterpenes (Eisenreich *et al.*, 2004).

The MEP pathway initiates with a transketolase-like decarboxylation reaction catalysed by the enzyme 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS) using pyruvate and glyceraldehyde-3-phosphate (GAP) as substrates to yield the first intermediate, 1-deoxy-D-xylulose-5-phosphate (Rohmer *et al.*, 1996; Lange *et al.*, 1998; Lois *et al.*, 1998). MEP is converted into a 5:1 mixture of IPP and DMAPP molecules through the action of six additional consecutive enzymatic reactions (Lange *et al.*, 2000; Rodríguez-Concepción and Boronat, 2002).

Diverse experimental evidence demonstrates that DXS catalyses a rate-controlling step in isoprenoid biosynthesis (Harker and Bramley, 1999; Kuzuyama *et al.*, 2000). The transcript of *dxs* accumulates in plant tissues with high isoprenoid demand, exemplified by tomato and pepper fruit ripening (Bouvier *et al.*, 1998; Enfissi *et al.*, 2005), gland trichomes of *Mentha piperita* (Lange *et al.*, 1998) and *Catharanthus roseus* (Chahed *et al.*, 2000), and young seedlings of *Arabidopsis thaliana* (Estévez *et al.*, 2000). Overexpression or reduction of DXS levels alters the amount of specific isoprenoids in *Arabidopsis* (Estévez *et al.*, 2001), tomato (Enfissi *et al.*, 2005), potato (Morris *et al.*, 2006), and *Ginkgo biloba* (Morris *et al.*, 2006). Thus, DXS is an obvious target for manipulation of this metabolic route.

Most enzymes of the MEP pathway are encoded by single-copy genes in flowering plants, whereas DXS is typically encoded by a small gene family (Walter *et al.*, 2002; Rodríguez-Concepción *et al.*, 2004; Phillips *et al.*, 2008; Cordoba *et al.*, 2009). Two related gene groups have been reported (Lange *et al.*, 1998; Krushkal *et al.*, 2003; Kim *et al.*, 2005). These gene classes display differential expression during development and in specific organs, suggesting a non-redundant function. Group I contains the *DXS1* (*CLA1*) gene from *Arabidopsis* and has been suggested to have predominantly a housekeeping function (Kim *et al.*, 2005). Group II includes genes such as *MtDXS2* from *Medicago truncatula* and *dxs2* from maize, whose expression correlates with and is essential for the accumulation of apocarotenoids during mycorrhization (Walter *et al.*, 2002; Floß *et al.*, 2008). Similarly *OsDXS3* from rice and both *PaDXS2A* and *PaDXS2B* from *Picea abies* have been proposed to be primarily involved in defence responses and secondary metabolism (Okada *et al.*, 2007; Phillips *et al.*, 2007). These findings led to the suggestion that the second class of DXS-type proteins might be dedicated to secondary metabolism. Members of a third DXS group have recently been reported. The expression pattern of the maize gene that belongs to this group correlates with carotenoid accumulation in seeds. However, the functionality and particular role of the proteins that group in this clade is still unclear (Vallabhaneni and Wurtzel, 2009).

Multiple signals modulate the DXS at the transcriptional and protein level (Cordoba *et al.*, 2009). In *Arabidopsis*, the transcription of *AtDXS1* is regulated by light, circadian rhythm, development, and sugars (Guevara-García *et al.*, 2005; Hsieh and Goodman, 2005). In addition, DXS protein levels are regulated post-transcriptionally in response to the fluctuation of product IPP and DMAPP levels (Guevara-García *et al.*, 2005) and also to the specific demand for particular isoprenoids (Rodríguez-Villalón *et al.*, 2009).

Because DXS is a flux-controlling enzyme for the MEP pathway, a deep understanding of the regulation of individual gene family members will underpin successful manipulation of this metabolic pathway. This is particularly relevant in agronomically important plants such as maize that represent a major food and potential biofuel source for some countries. The need to elucidate further the maize *dxs* family motivated this study. In this work a detailed analysis of the *dxs* family from maize is reported. In addition, to extend previous work, new data are provided that show that each of the maize *dxs* genes represents a different phylogenetic clade. These *dxs* genes encode functional DXS proteins with a plastid subcellular localization. The maize *dxs* genes display a particular tissue-specific expression that changes during plant development and in response to external signals. It is also shown that DXS protein levels increase in tissues of high IPP and DMAPP demand, including photosynthetic tissues and yellow kernels. The results demonstrate that the abundance of maize DXS protein is modulated by post-transcriptional regulation in response to the alteration of pathway flow, similar to the situation in *Arabidopsis*. This observation demonstrates that the post-transcriptional regulation of *dxs* is highly conserved in evolution and represents a major mode of regulation of this enzyme.

Materials and methods

Plant material and growth conditions

Experiments with mature plants were carried out with certificated inbred lines of *Zea mays*; Oso for vegetative and reproductive tissues (Monsanto Inc.), and CML311 (CINVESTAV-Irapuato) and Wisconsin 23 for ears grown in greenhouse conditions in a soil mixture of vermiculite:peat moss (1:1). Seedlings were obtained from the FR992×FR697 hybrid line in a growth chamber at 24 °C under a 16:8 h light:dark photoperiod or in complete darkness for etiolated seedlings. For root growth kinetics, seedlings were grown in darkness at 24 °C over paper saturated with water. For light induction 12-day-old dark-grown seedlings were shifted to light for the indicated times. For *in vitro* grown plants; seeds were germinated on 1× (GM) Murashige and Skoog basal salts (Gibco BRL, Grand Island, NY, USA), 1% (w/v) sucrose, 1× Gamborg's vitamin solution (Sigma-Aldrich, Inc., St Louis, MO, USA), 0.05% (w/v) MES, with 0.8% (w/v) phytoagar® (Gibco BRL). For fosmidomycin and norflurazon treatments, seeds were germinated for 7 d and transferred to either GM or GM supplemented with 100 μM fosmidomycin (kindly provided by Drs Shinjiro Yamaguchi and Yuji Kamiya, from the Plant Science Research Center RIKEN) or 5 μM norflurazon for 5 d.

Cloning of the dxs1, dxs2, *and* dxs3 *genes*

A *dxs* 911 bp genomic fragment was amplified by PCR using total DNA and the oligonucleotides RSF and RSR (Table 1). The PCR

product was cloned into the pBlue Script vector (Invitrogen™, Carlsbad, CA, USA) and sequenced. Related *dxs* sequences were obtained from a BLAST search (Altschul *et al.*, 1997), and this information was used to search repositories of cDNAs. The *dxs1* sequence was obtained from a clone (accession no. CF649815) from the maize database (http://www.maizegdb.org/). This expressed sequence tag (EST) was sequenced and deposited in GenBank (accession no. AY951981). The sequence for the *dxs2* type was obtained by PCR amplification from genomic DNA using the primers ZmDXS2F-5' and ZmDXS2R-TGA (Table 1). The *dxs3*-type cDNA was amplified from ears, using the primers ZDXS3F-GTW and DXS3'R (accession no. HQ113384). The identity of each clone was confirmed by sequencing.

The rapid amplification of cDNA ends (RACE) was done using the First Choice® RLM-RACE kit, following the manufacturer's instructions (Ambion Inc., Austin, TX, USA), using 3 μg of a mix of total RNA from ears (4 cm long) and roots. For cDNA synthesis, two specific primers, ZmDXS2R-TGA or ZDXS2R-GTW, were used. Nested amplifications were done using the reverse primers DXS2-5RACERv and DX2-5RC-In in combination with 5′ RACE inner primer (supplied by Ambion). The amplified products were cloned in pCR®II TOPO® vector (Invitrogen) and 10 independent clones were sequenced.

Phylogenetic analysis

The virtual translations of *dxs* sequences were aligned using Clustal W (Thompson *et al.*, 1994). The putative chloroplast transit peptide was excluded from all the sequences and no further adjustment was used to perform the phylogenetic analysis. A Neighbor–Joining tree was constructed using MEGA version 4 (Tamura *et al.*, 2007) with a bootstrap of 1000 replicates. The final phylogenetic tree was drawn using the TreeView program (Page, 1996).

Genomic Southern analysis

High molecular weight DNA was isolated from etiolated maize leaves. A 20 μg aliquot of total DNA was digested with *Bam*HI, *Pst*I, *Eco*RI, or *Hin*dIII endonucleases. DNA was size-fractionated by electrophoresis in 0.8% agarose gels and transferred to Hybond N^+ nylon membrane (GE, Amersham Biosciences, UK). Hybridizations were performed at 65 °C overnight using a 911 bp genomic fragment of *dxs2* as the ^{32}P-radiolabelled probe (Fig. 1A) and washed at 65 °C with 0.1% (w/v) SDS, 0.1% (w/v) SSC (Church and Gilbert, 1984).

Expression analysis

Total RNA was isolated as previously described (Logemann *et al.*, 1987), fractionated by electrophoresis on 1.5% (w/v) formaldehyde–agarose gels, and then transferred onto a Hybond N^+ nylon membrane (GE). Hybridizations and washes were carried out as for those described for the Southern analysis. The probes used correspond to a 318 bp cDNA for *dxs1* obtained with the ZDXS1F and ZDXS1R-GTW primers and a 221 bp fragment from the 3′end of *dxs2* (Fig. 1A, Table 1). For *dxs3* a 355 bp fragment was used with the primers DXS3F and DXS3'R (Table 1).

The cDNA was synthesized from 2 μg of DNase-treated total RNA using Moloney murine leukaemia virus reverse transcriptase (Invitrogen) and the ZDXS2R-GTW or poly(dT) primers, following the manufacturer's instructions. For PCR, 2 μl of both reverse transcription products were amplified using High Fidelity Taq DNA Polymerase (Invitrogen). Primers used included: for *dxs2*, ZDXS2F5GW2 (for type 1) or ZDX2FGwC1 (for type 2) and ZDX2RvI60; for *dxs3*, DXS3F andDXS3'R; and for ubiquitin, Ubq-F and Ubq-R, as control (Table 1).

Functional complementation

The cDNAs of the three different *dxs* clones from maize were inserted into the plasmids pCMV for *dxs1*, pGEM-Teasy (Promega, Madison, WI, USA) for *dxs2*, and pTOPO-TA (Invitrogen) for *dxs3*. A pBluescript plasmid with the full-length *dxs* sequence from *Arabidopsis* and an empty vector were used as controls. These constructs were transformed into the EcAB4-2 *Escherichia coli* strain, defective in DXS activity (Sauret-Güeto *et al.*, 2006*b*). Transformants were selected on Luria–Bertani (LB) broth plates supplemented with 1 mM mevalonate and the appropriate antibiotics. Two colonies per construct were grown on plates supplemented with antibiotics but no mevalonate. Single colonies were then inoculated into 10 ml of LB medium with no mevalonate and grown at 37 °C for 15 h and 40 h at 150 rpm. Bacterial growth was recorded by measuring the OD_{600} of the culture directly (when <1.0 OD) or diluted (when >1.0 OD).

Western blot analysis

Total protein samples were obtained from different tissues in SDS sample buffer [0.125 M TRIS-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol]. The protein concentration was determined with Bradford reagent (Bio-Rad, Hercules, CA, USA). A 10 μg aliquot of total protein was used for protein analysis, separated by SDS–PAGE. To verify equal protein loading, a parallel gel was run and stained with Coomassie Brilliant Blue R-250. The proteins were transferred onto nitrocellulose (Hybond C, GE) by electroblotting. Immunodetection was performed using a 1:1000 dilution of the polyclonal antibodies against the *Arabidopsis* DXS protein (Estévez *et al.*, 2000) and detected using the BCIP/NBT substrate kit (Zymed Laboratories).

Subcellular localization

The 5′ *dxs* fragments containing the putative transit peptide were cloned in pENTR/D/TOPO vector (Invitrogen™). The primers used were: ZDXS1F-GTW and ZDXS1R-GTW for *dxs1* (106 amino acids); ZDXS2F5GW2 and ZDXS2R-GTW for *dxs2* T1 (114 amino acids, DXS2); ZDXS2M-GW and ZDXS2R-GTW for *dxs2* T2 (52 amino acids, DXS2s); and ZDXS3F-GTW and ZDXS3R-GTW for *dxs*3 (124 amino acids). These fragments were fused to green

Table 1. Nucleotide sequences of gene-specific primers

Gene	Primer name	Sequence
dxs1	ZDXS1F	5′-ATGGCTCTGTCGACGTTCTCTGTCC-3′
	ZDXS1R-GTW	5′-CTTGGAGACGTGGAAGATGAC-3′
	ZDXS1F-GTW	5′-CAC-CATGGCTCTGTCGACGTTCTC-3′
dxs2	RSF	5′-TATCCGCACAAGATCCTGAC-3′
	RSR	5′-TGCTGTTCCGCGATGCCGACA-3′
	ZmDXS2F-5′	5′-ATGGCGCTCCAGGCATCGTCGTCG-3′
	ZmDXS2R-TGA	5′-TCACTTGAGCTGCATGGCC-3′
	ZDXS2F5GW2 (F1)	5′-CACCATGGCGCTCCAGGCATCGTCG-3′
	ZDXS2R-GTW	5′-CTTGGACACGGTGTGCACGAC-3′
	DXS2-5RACERv	5′-TCTTCATGTGGAGCGGGTAGT-3′
	DX2-5RC-In	5′-CGATCTTCCACGGTCCCGA-3′
	ZDX2FGwC1 (F2)	5′-CACCTGTGGTGCCGGTGCTCATGC-3′
	ZDX2RvI60 (R1)	5′-GATCTTCCACGGTCCCGACG-3′
	ZDXS2M-GW	5′-CACCATGATGGTTTCCAAGGAGCCGGCG-3′
dxs3	ZDXS3F-GTW	5′-CACCATGGACACGGCGTTTCTGAG-3′
	DXS3′R	5′-ACAAAACTAAGTAGCTTTATTACA-3′
	DXS3F	5′-TTGATGAAGTAGGGGAAGGAACC-3′
	ZDXS3R-GTW	5′-ACACGGTTGGCATTTTCTGGA-C-3′
Ubq	Ubq-F	5′-CACTTGGTGTTGCGTCTCAG-3′
	Ubq-R	5′-CACCTCAAGGGTGATCGTCT-3′

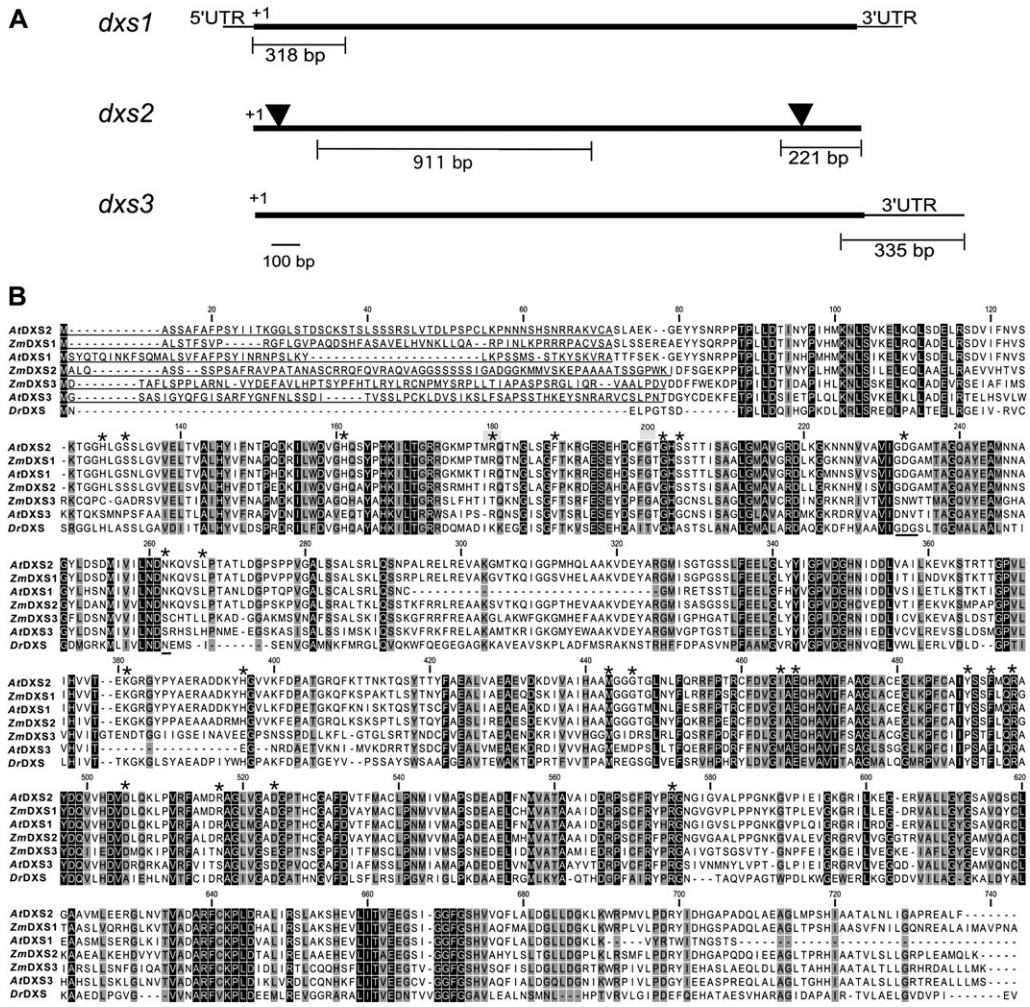

Fig. 1. Three *dxs* genes exist in maize. (A) Diagram of the *dxs1*, *dxs2* and *dxs3* clones used in this work showing the translation start site (+1), the positions of introns (triangles), and the fragments used as probes (lines). (B) Multiple alignment of the *Zea mays* (*Zm*) deduced DXS1, DXS2, and DXS3 protein sequences compared with the DXS proteins from *Arabidopsis thaliana* (*At*) and *Deinococcus radiodurans* (*Dr*). Amino acid identity is marked in grey. The sequence predicted by the ChloroP program as the plastid transit sequence for each protein is underlined. Residues in the active site are marked with an asterisk above (*). The transketolase consensus thiamine pyrophosphatase (TPP)-binding domain start and end is underlined.

fluorescent protein (GFP) using the pEarlyGate103 binary vector (Earley *et al.*, 2006). The resulting constructs (20 µg) were introduced by polyethylene glycol (PEG)-mediated transformation into maize mesophyll protoplasts following the protocol reported by Sheen's laboratory. GFP expression was observed using confocal META laser scanning microscope (Carl Zeiss, LSM510), equipped for excitation with an argon (Ar2) 488 nm laser.

Results

Isolation of the maize dxs *genes*

To characterize the DXS family in maize, a region conserved between the *DXS1* (*CLA1*) gene from *Arabidopsis* (At4g15560) and the *dxs* gene from bacteria (Mandel *et al.*, 1996; Sprenger *et al.*, 1997; Hahn *et al.*, 2001) was used to identify several putative *dxs*-like genes in the maize genomic database (http://www.maizegdb.org/). *In silico* analysis showed three different *dxs* types among the sequences obtained. These data agree with previous reports on the existence of multiple *dxs* genes (Walter *et al.*, 2002; Vallabhaneni and Wurtzel, 2009).

The full *dxs1* gene was obtained by sequencing an EST available in the maize database (GenBank accession no. AY951981). This EST contains the complete open reading frame (ORF) plus 140 bp of its 5'-untranslated region (UTR) and 125 bp of the 3'-UTR sequences (Fig. 1A). The second *dxs* gene (designated as *dxs2*) was initially identified from a partial clone of 911 bp obtained through direct amplification by PCR from maize genomic DNA (GenBank accession no. AY944061). As the sequence of this fragment displays 100% identity with a sequence deposited in the maize database (CG162236 and AY946270), a hypothetical contig was constructed. Based on this reconstruction, specific primers were designed and a 2.1 kb PCR product from genomic DNA was amplified. The PCR product

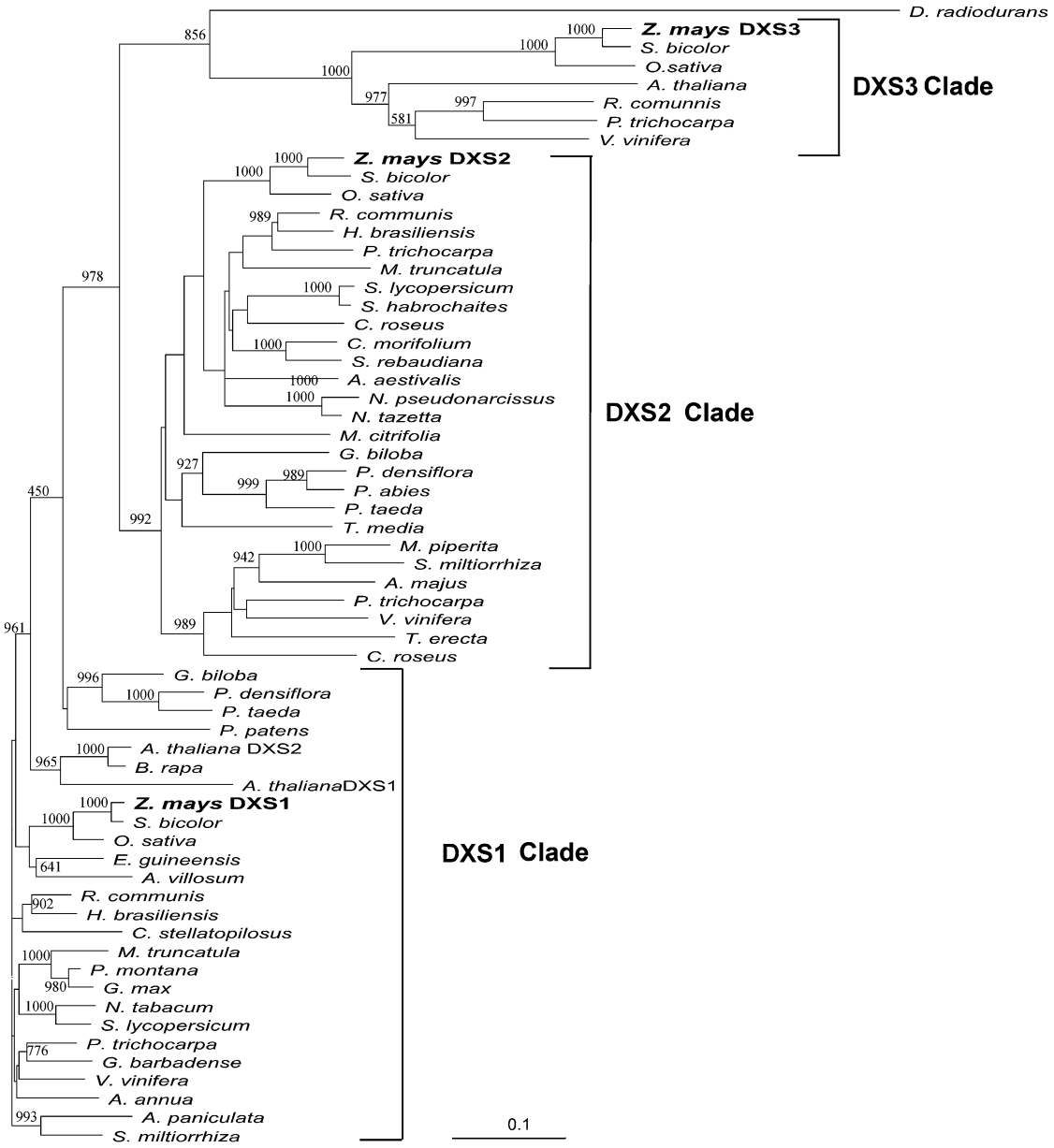

Fig. 2. A phylogenetic tree was constructed using the following sequences: *Adonis aestivalis* (ABK35283.1); *Amomum villosum* (ACR02668.1); *Andrographis paniculata* (AAP14353.1); *Arabidopsis thaliana* (DXS1 clade DXS/CLA1 At4g15560, DXLS2/DXL1 At3g21500, DXS3 clade DXS3/DXL2 AT5G11380); *Artemisia annua* (AAD56390.2); *Antirrhinum majus* (AAW28999.1); *Brassica rapa* (ABE60813.1); *Catharanthus roseus* (ABI35993.1, CAA09804.2); *Chrysanthemum x morifolium* (BAE79547.1); *Croton stellatopilosus* (BAF75640.1); *Elaeis guineensis* (AAS99588.1); *Glycine max* (ACO72582.1); *Ginkgo biloba* (DXS1 clade AAS89341.1, DXS2 clade AAR95699.1); *Gossypium barbadense* (ABN13970.1); *Hevea brasiliensis* (DXS1 clade AAS94123.1, DXS2 clade ABF18929.1); *Medicago truncatula* (DXS1 clade CAD22530.1, DXS2 clade CAN89181.1); *Mentha piperita* (AAC33513); *Morinda citrifolia* (AAL32062.1); *Narcissus pseudonarcissus* (CAC08458.1); *Narcissus tazetta* (ADD82535.1); *Nicotiana tabacum* (CBA12009.1); *Oryza sativa* (DXS1 clade NP_001055524, DXS2 clade NP_001059086, DXS3 clade BAA83576); *Physcomitrella patens* (XP_001756357.1); *Picea abies* (ABS50519.1); *Pinus densiflora* (DXS1 clade ACC54557.1, DXS2 clade ACC54554.1); *Pinus taeda* (DXS1 clade ACJ67021.1, DXS2 clade ACJ67020.1); *Populus trichocarpa* (DXS1 clade XP_002312717.1, DXS2 clade XP_002303416.1, XP_002331678.1, DXS3 clade XP_002308644.1); *Pueraria montana* (AAQ84169.1); *Ricinus communis* (DXS1 clade XP_002516843.1, DXS2 clade XP_002533688.1, DXS3 clade XP_002514364.1); *Salvia miltiorrhiza* (DXS1 clade ACF21004.1, DXS2 clade ACQ66107.1);

contained the entire mature DXS2 ORF (Fig. 1A), but apparently lacked part of the transit peptide for the plastid localization. An intron of 116 bp was identified in the 3′ region (Fig. 1A). To determine the 5′ terminus of the gene transcript an RNA ligase-mediated RACE (RLM-RACE) was performed using RNA from roots and immature ears. Based on the sequence of 10 independent clones, two major transcription start sites for *dxs2* were identified: one is located within a putative intron (Fig. 7B) and the other is identical to a recently published *dxs* sequence (Vallabhaneni

and Wurtzel, 2009). Since the RLM-RACE relies on the cDNA synthesis from specific linkers that ligate only to transcripts that contained the CAP structure, it was concluded that both species are independent initiation sites and represent different *dxs2* primary transcript species.

Finally, the complete cDNA sequence (2515 bp) of the third gene was obtained through RT-PCR also based on a hypothetical contig from the maize database. This cDNA contains the entire ORF of the *dxs3* gene and 346 bp of the 3′-UTR (Fig. 1A).

The deduced ORFs from the three genes, *dxs1*, *dxs2*, and *dxs3*, consist of 719, 723, and 722 amino acid residues, respectively (Fig. 1B). The overall similarity between the three DXS proteins ranges between 69% and 47%, with DXS3 being the most divergent. The three DXS proteins have conserved the three domains characteristic of the DXS family. Residues in the proteins that have been reported as crucial for catalysis in *Deinococcus radiodurans* such as glutamate (DXS1, 445; DXS2, 453; DXS3, 453), arginine (DXS1, 473; DXS2, 481; DXS3, 481) and arginine (DXS1, 553; DXS2, 561; DXS3, 561) are conserved in the three maize DXS protein types (Xiang *et al.*, 2007). Similar to other DXS proteins, the consensus thiamine pyrophosphate (TPP)-binding domain is highly conserved between DXS1 and DXS2 but only partially conserved in DXS3. Some amino acids that participate in the active site such as histidine (DXS1, 110; DXS2, 118), tyrosine (DXS1, 467; DXS2, 474), and aspartate (DXS1, 502; DXS2, 510), apparently involved in binding the GAP molecule, are conserved in DXS1 and DXS2 but not in DXS3 (Xiang *et al.*, 2007). These differences are consistent with the DXS3 group being the most divergent of the three. They also open up the question of the functionality of this protein and its affinity for the GAP molecule.

The DXS maize family belongs to three independent phylogenetic groups conserved among plants

To obtain a better picture of the relationships of the maize DXS proteins relative to those from other organisms, a Neighbor–Joining tree was constructed. The tree included representative proteins from different plants, as well as that from *D. radiodurans*, as a reference (Fig. 2). The present analysis includes only sequences that contain the complete DXS ORF. The phylogenetic tree branches into three independent groups of DXS proteins conserved among plants. Clade DXS1, previously referred to as DXS1 (Walter *et al.*, 2002), includes proteins from a variety of dicots and monocots and the well characterized DXS1/CLA1 from *Arabidopsis*. This group also contains a DXS-like protein sequence (DXS2/DXL1) from *Arabidopsis* (At3g21500), a protein whose function is still unknown (Phillips *et al.*, 2008). A second clade (DXS2) in this tree includes maize DXS2 together with representatives from *M. truncatula*, *C. roseus*, and *M. piperita*, among others. Members within this group have been shown to be essential for the synthesis of particular isoprenoids involved in secondary metabolism, including apocarotenoids (Floß *et al.*, 2008). Finally, a third clade (DXS3) includes proteins such as maize DXS3, rice DXS2, and *Arabidopsis* DXS3/DXL2 (At5g11380). This group is conserved among plants species, but it is the more distant phylogenetic DXS group. The DXS protein from *D. radiodurans* clusters in a separate clade from plant DXS proteins.

The three maize dxs *genes encode functional proteins*

To determine whether maize DXS proteins are functional enzymes, complementation experiments were performed using an *E. coli* strain EcAB4-2 defective in the DXS gene. This strain contains heterologous genes that permit the use of supplied mevalonate to sustain growth in the absence of DXS (Campos *et al.*, 2001). In the absence of exogenous mevalonate this strain cannot grow unless a functional DXS protein is introduced. The EcAB4-2 strain was transformed with each of the three maize *dxs* cDNAs expressed from a bacterial promoter. Transformants harbouring DXS1 grew overnight on plates without mevalonate, similar to control bacteria expressing *Arabidopsis* DXS. Those harboring DXS2 and DXS3 grew slowly and were only visible after 2 d of culture (Fig. 3). Control strains transformed with an empty plasmid were unable to grow in the absence of mevalonate even after longer incubation times. From this assay it was confirmed that the three maize *dxs* genes encode functional DXS proteins.

Expression analysis of the dxs1, dxs2, *and* dxs3 *genes in maize plants tissues*

To better define the expression pattern of each maize *dxs* gene, a northern survey of nine different maize organs was conducted. As shown in Fig. 4A, the *dxs1* transcript

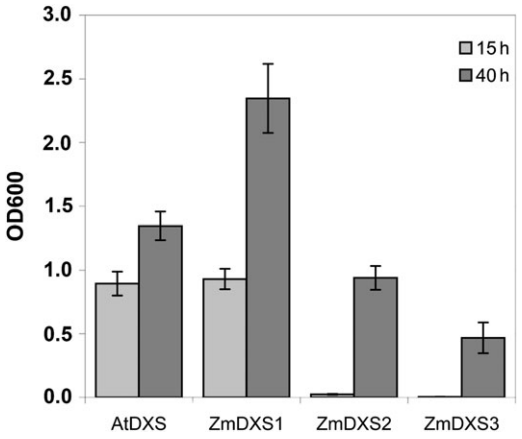

Fig. 3. Functional complementation of the DXS maize proteins in *E. coli*. The *E. coli* strain EcAB4-2 defective in DXS activity was complemented with each of the three maize *dxs* genes and grown in the absence of mevalonate. Growing capacity was followed by optical absorbance at 600 nm at 15 h and 40 h. The *DXS/CLA1* gene from *Arabidopsis* was used as a positive control. No growth was observed at these times in the empty vector, used as a negative control. Mean and standard error of a total of six cultures (n=6) from three separate experiments are represented.

accumulated predominantly in green tissues including leaves, green husks, and immature tassel. By far the highest expression level detected for this gene was in young leaves, and it substantially decreased in leaves from mature soil-grown plants. Low *dxs1* levels were found in immature ears, roots (Fig. 4A), and kernels, independent of whether they were white or yellow (Fig. 4B).

The other two *dxs* genes were expressed at lower levels than *dxs1* in all tissues analysed. *dxs2* transcript was detected readily in mature leaves, roots (Fig. 4A, C), and in yellow kernels (Fig. 4B). *dxs2* transcript was barely detectable in white kernels and undetectable in all the other tissues analysed. The *dxs3* transcript was detected in all tissues analysed, but the highest levels were observed in mature leaves, immature ears, and husks (Fig. 4A, C).

Expression analyses of the dxs1 *and* dxs2 *genes during leaf development*

In several species, the levels of *dxs* transcripts have been observed to change during plant development. To address this in maize, the transcript levels of the three *dxs* genes

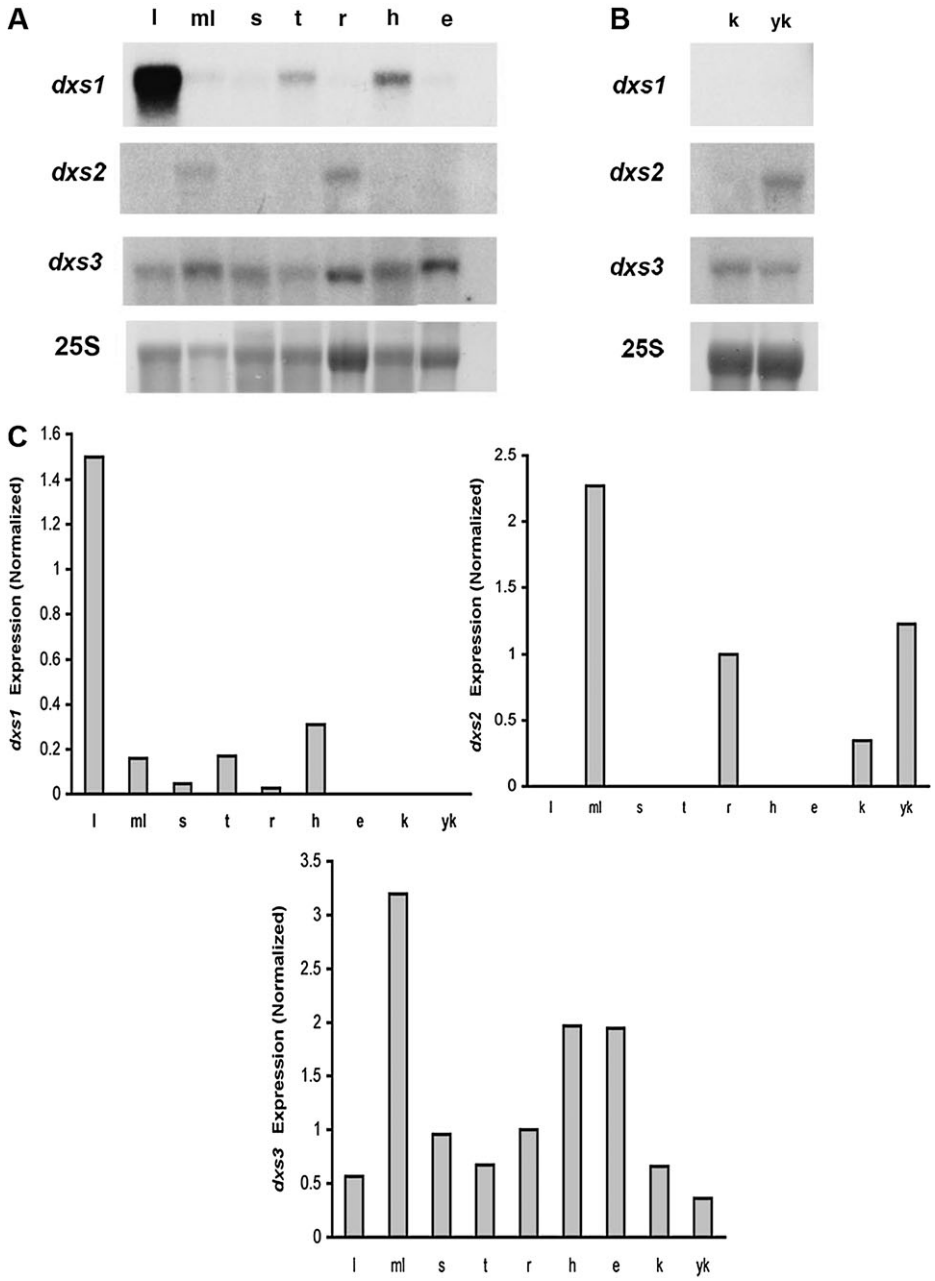

Fig. 4. Transcript accumulation of the three *dxs* genes in maize tissues. (A) Northern blot analysis of total RNA extracted from 8-day-old leaves (l), 2- month-old mature leaves (ml), silks (s), tassel (t), roots (r), husks (h), and immature ears (e). (B) Mature kernels from white (k) or yellow (yk) varieties. Each lane contains 20 μg of RNA. Northerns were hybridized with specific probes for *dxs1*, *dxs2*, and *dxs3* genes. The 25S rRNA is shown as a loading control (25S). (C) Densitometric quantifications of the *dxs1*, *dxs2*, and *dxs3* transcript levels shown in A and B, relative to the corresponding 25S control for each lane.

were followed during seedling development. Total mRNA from the first and second leaves was isolated from 8-, 12-, and 20-day-old plantlets and the expression of these genes analysed. As shown in Fig. 5A, the abundance of the *dxs1* transcript was higher in younger leaves than in older leaves. *dxs2* and *dxs3* transcripts were barely detected at these developmental stages (data not shown).

Maize leaf blades have Kranz anatomy, a characteristic of C_4 plants (Nelson and Langdale, 1989). C^4 differentiation develops in a gradient from the tip to the base of the blade and to a lesser extent in the sheath region which are primarily C_3 organs. To analyse whether the *dxs1* transcript expression profile displays any differences in these tissues, total RNA from leaf sheath and leaf blade was extracted independently from first and second leaves of 20-day-old seedlings. As shown in Fig. 5B, the *dxs1* gene transcript accumulated to higher levels in the sheath of the first and second leaves than in their corresponding blades. The highest *dxs1* expression was detected in the youngest tissue (third leaf), which, due to its size, was not separated between blades and sheath. These data show that *dxs1* is preferentially expressed in the sheaths, which are anatomically more C_3-like tissues.

The initial analysis showed that the highest *dxs2* expression was in roots (Fig. 4A, C). Previous reports have shown that this gene plays a role in the biosynthesis of specific carotenoids in mycorrhizal colonized roots (Walter *et al.*, 2000, 2002). The expression of this gene was followed in 4- to 20-day-old seedling roots. As shown in Fig. 5C, the *dxs2* transcript accumulated to higher levels in the 12- and 15-day-old roots, followed by a significant (12-fold) decline in later developmental stages.

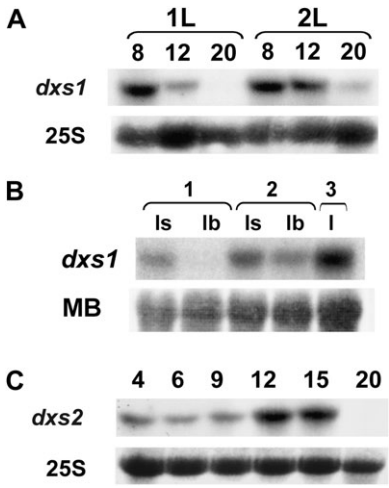

Fig. 5. The levels of *dxs* genes change during maize development. (A) Northern blot analysis of total RNA from first (1L) and second (2L) leaves of 8-, 12-, and 20-day-old maize seedlings. (B) RNA blot of 20-day-old maize leaf sheath (ls), leaf blade (lb), and whole leaf (l) tissues. The leaf number from which each tissue was extracted is marked: (1) first, (2) second, and (3) third leaf. (C) Northern blot analysis of total RNA from 4-, 6-, 9-, 12-, 15-, and 20-day-old maize seedling roots. Each lane contains 20 μg of RNA and the membranes were hybridized sequentially with different probes. The hybridization of the 25S rRNA or methylene blue- (MB) stained gels are also shown as the loading controls.

Light regulation of the dxs *gene transcripts*

Light plays a major role in the regulation of the expression of several *dxs* genes in various species (Mandel *et al.*, 1996; Hsieh and Goodman, 2005). Therefore, to analyse the effect of light on the accumulation of the *dxs* maize gene transcripts, total RNA was extracted from 8-day-old leaves, grown under constant darkness followed by light exposure. The *dxs1* transcript rapidly accumulated upon 1 h of illumination, and continued for up to 21 h, with a final increase of 5-fold above the initial level (Fig. 6A). These responses correlated with the light induction observed for the *rbcS* photosynthetic gene (Fig. 6A). Although the *dxs2* gene was expressed at lower levels than *dxs1* in leaves, light exposure resulted in a transient accumulation (2-fold) after 5 h of illumination (Fig. 6A). In contrast, for *dxs3*, only a minor change on the transcript level was observed after light exposure (Fig. 6B).

Multiple transcripts exist for the dxs2 *gene*

Two major transcript species were identified for the *dxs2* gene by RACE analysis. The sequence of independent clones showed that all of them are identical in their 3′

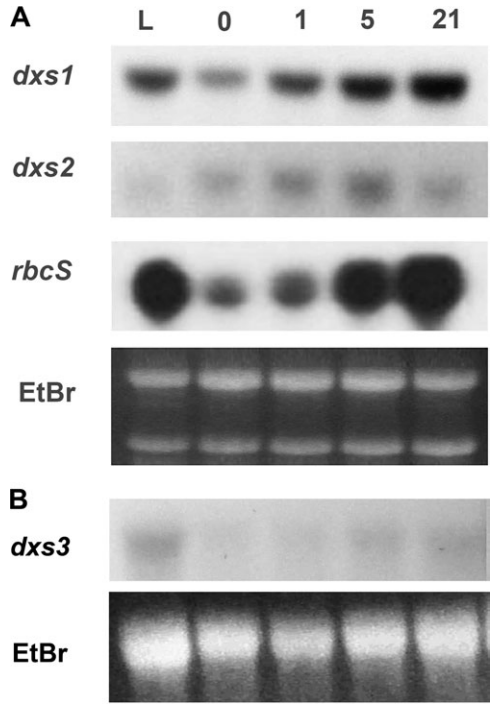

Fig. 6. Light regulates the expression of *dxs* maize genes. Twelve-day-old seedlings were grown under darkness and then exposed to light for 0, 1, 5, and 21 h or grown in a 8:16 light:dark cycle (L). Each lane contains 10 μg (A) or 20 μg (B) of total RNA. (A) The same membrane was hybridized against *dxs1*, *dxs2*, and *rbcS* or (B) *dxs3* probes. The *rbcS* gene expression was included as a control of a light-regulated gene. The ethidium bromide- (EtBr) stained gels are shown as the loading controls.

termini and in the mature protein but have different sequence at their 5′ termini. The region where these differences were found encoded part of the putative plastid transit peptide (Fig. 7). Consequently, the resultant proteins differ in ~30 amino acids at their N-termini. One possible explanation for these results is that each transcript species derives from independent *dxs2* genes. To test this, a Southern blot of maize genomic DNA digested with four restriction endonucleases was hybridized against a specific probe for the *dxs2* gene in a region common to all of the RACE transcripts. A single prominent band for each enzyme was detected (Fig. 7A). The weak hybridization to additional bands observed in some lanes probably corresponds to the related *dxs1* and *dxs3* genes. It is concluded that in the FR992×FR697 inbred line analysed there is only one *dxs2*, and by inspection of the B73 genome sequence only a single gene copy was also found, located on chromosome 7.

Close inspection of the genomic region from the maize data bank (http://www.maizegdb.org) that displays 100% identity to the present EST (gi 226373747, gbAC231619.3) showed that sequences identical to each of the two major 5′ termini of the RACE products exist upstream of the reading frame (Fig. 7B). The sequence from the 5′ termini of transcript type 1 (T1) is present ~400 bp upstream of the 5′ termini of transcript type 2 (T2). An intron prediction

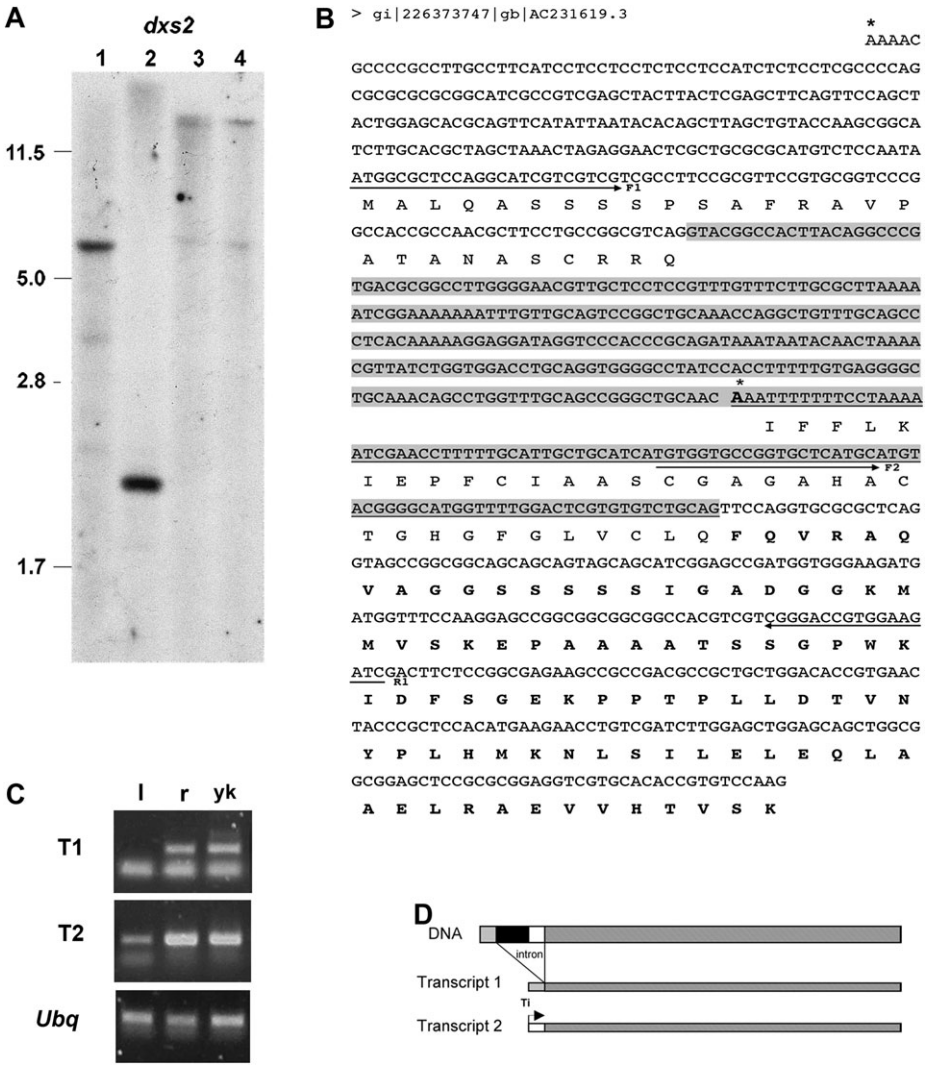

Fig. 7. Two different transcript species exist for the *dxs2* maize gene. (A) Southern analysis of the *dxs2* gene. A 20 μg aliquot of total DNA from *Z. mays* (L.: FR992×FR 697) was digested with *Bam*HI (lane 1), *Pst*I (lane 2), *Eco*RI (lane 3), and *Hin*dIII (lane 4) restriction enzymes. Hybridization was performed under stringent conditions as described in the Materials and methods. The probe used corresponds to a 911 bp genomic fragment for the *dxs2* gene, as show in Fig. 1A. (B) Genomic sequence of the 5'-terminal region of the *dxs2* transcripts type 1 and 2. The sequence includes the predicted intron marked in grey. The primers used for the *dxs2* PCR amplification are marked by the arrows (F1 or F2 and R1). The asterisk (*) marks the transcription initiation sites obtained from the RACE for the two transcript types. (C) RT-PCR from leaves (l), roots (r), and yellow mature kernels (yk). To access the specific *dxs2* transcripts, PCRs from each cDNA were obtained using the same reverse primer (R1) but different 5' oligonucleotides: F1 for type 1 (T1) and F2 for type 2 (T2). RT-PCRs from the ubiquitin (Ubq) from the same RNAs are shown as a control. (D) Diagram of the genomic region and the two different *dxs2* transcript types whose only difference is the very 5' sequence.

program identified a putative 359 bp intron within this region that, when spliced, will generate the exact sequence present in the transcript T1 (Fig. 7d). In contrast, the unique sequence present at the 5′ termini of the transcript T2 is part of the predicted intron (Fig. 7d). From these data it is concluded that the *dxs2* gene is transcribed from two different sites located ~600 bp apart.

To determine if these transcript species differentially accumulate in particular tissues, RT-PCR analysis was done using cDNA generated from the ZDXS2R-GTW oligonucleotide from those tissues where the *dxs2* transcript was detected. Specific primer pairs that distinguish each transcript species were used. As shown in Fig. 7C, the transcript that starts within the intron (T2) accumulated in the three tissues and apparently at higher levels than T1. In contrast, the transcript T1 was only detected in kernels and roots but not in cDNA from mature leaves. Such differences cannot be attributed to the quality of RNA, which appears to be similar judged from the ubiquitin levels used as control.

The maize DXS proteins are targeted to plastids

The software ChloroP (http://www.cbs.dtu.dk/services/ChoroP) and Predotar (Emanuelsson *et al.*, 1999; Small *et al.*, 2004) predicted a chloroplast transit sequences of 52 amino acids for DXS1 and 67 for DXS3 (Fig. 1B). For DXS2, a transit peptide of 67 amino acids was predicted for the protein encoded in the transcript T1. The transit peptide predicted from T2 is relatively short, with only 17 amino acids, if starting from the first methionine after the initiation start site (Fig. 7B, D). *In silico* this peptide is not recognized as a canonical chloroplast transit peptide. To analyse the subcellular localization of the different maize DXS isoforms, each was fused to a GFP. These fusions were introduced into maize mesophyll protoplasts and the GFP fluorescence analysed by confocal imagining (Fig. 8). The GFP fluorescence of the fusions DXS1–GFP, DXS3–GFP, and DXS2–GFP derived from the T1 transcript was observed exclusively inside chloroplasts (Fig. 8A, D, J). These localizations were confirmed by the co-localization of GFP with chlorophyll autofluorescence. In contrast, the fluorescence of the DXS2 fusion derived from the T2 transcript accumulated exclusively in the cytosol (Fig. 8G). These results confirm the prediction that the putative protein encoded by the transcript T2 does not localize to chloroplasts.

Expression and regulation of the maize DXS proteins

To analyse protein accumulation in several maize organs, heterologous antibodies against the *Arabidopsis* DXS protein were used. As shown in Fig. 9A, a protein with a similar molecular mass to that of the *Arabidopsis* DXS was detected predominantly in (9-day-old) young leaves and in yellow kernels. This protein was also detected at lower levels in all five other tissues analysed including husks, tassel, and roots. The capacity of the antibody to recognize the maize DXS1 and DXS2 proteins was demonstrated by western analysis after protoplast expression assays (data not shown).

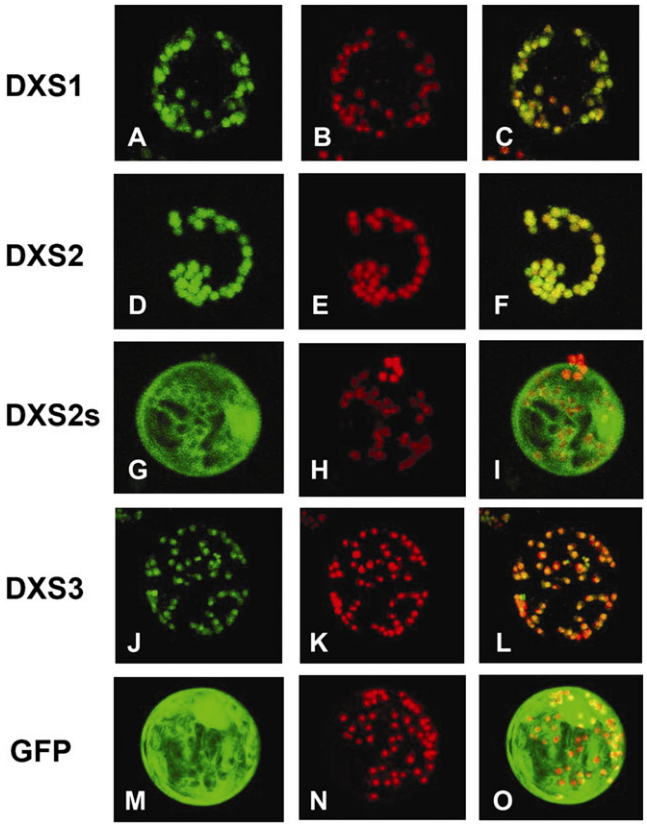

Fig. 8. Subcellular localization of the ZmDXS proteins. The N-termini of the proteins encoded by the *dxs* genes were fused to GFP using 106 amino acids for DXS1, 114 amino acids for DXS2 (from transcript type 1), 52 amino acids for DXS2 (from transcript type 2), and 124 amino acids for DXS3 containing the putative chloroplast transit peptides. Mesophyll protoplasts were transformed with each GFP fusion construct containing DXS1 (A), DXS2 (D), DXS2s (G), DXS3 (J), and GFP (M). The GFP vector (M–O) was used as a control. Confocal microscope images of the GFP fluorescence (A, D, G, J, and M), chlorophyll autofluorescence (B, E, H, K, and N), and the merged images for GFP and chlorophyll for each construct (C, F, I, L, and O) are shown.

In *Arabidopsis*, the DXS protein accumulates after application of inhibitors of this pathway, probably as a response to decreased levels of the MEP pathway final products (IPP and DMAPP) (Guevara-García *et al.*, 2005; Sauret-Güeto *et al.*, 2006*a*). To explore whether this feedback mechanism is conserved in maize, the accumulation of DXS protein was analysed in seedlings grown *in vitro* and treated with the herbicide fosmidomycin. Fosmidomycin inhibits the activity of the second enzymatic step of the pathway, and as a consequence the levels of IPP and DMAPP are reduced (Kuzuyama *et al.*, 1998; Schwender *et al.*, 1999; Laule *et al.*, 2003). Seven day-old maize seedlings were transferred to media with or without 100 μM fosmidomycin for 5 d and the level of the DXS protein was estimated in the newly emerging leaves that are pale green (Fig. 9B). The DXS protein consistently accumulated to higher levels (~10 times) in the

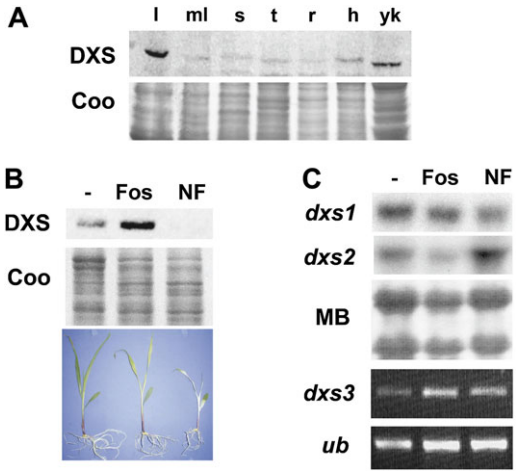

Fig. 9. Accumulation of the DXS protein in different maize tissues. (A) Expression of the DXS protein in maize tissues. Total protein extracts from 8-day-old leaves (l), 2-month-old mature leaves (ml), silks (s), tassel (t), roots (r), husks (h), and mature yellow kernels (yk) were used to perform an immunoblot analysis with heterologous antibodies against *Arabidopsis* DXS. A specific band of similar molecular mass to the *Arabidopsis* DXS protein is observed in maize tissues. Coomassie brilliant blue- (Coo) stained gels were used as loading controls. (B) Expression of DXS protein in maize seedlings treated with fosmidomycin and norflurazon. Maize seedlings were grown for 7 d on MS medium. Treatment was done by transfer to MS medium for control (–), or MS medium supplemented with 100 μM fosmidomycin (Fos) or 5 μM norflurazon (NF). Samples were collected 5 d after treatment. A 10 μg aliquot of total protein extracts from the third leaves was loaded in each lane. The level of the DXS protein was analysed by protein gel blots as described in A. Coomassie brilliant blue-stained gels were used as loading controls (Coo). (C) Relative levels of expression of the *dxs* genes after fosmidomycin and norflurazon treatments. RNA was extracted from the same samples used for the protein gel blot analysis in B. Northern blot was used to analyse *dxs1* and *dxs2*. Each lane contains 5 μg of total RNA from each condition. The methylene blue-stained membrane (MB) is shown as a loading control. RT-PCR was used to analyse the *dxs3* gene expression. *Ubiquitin* (*Ub*) expression was used as loading control.

presence of fosmidomycin (Fig. 9B). This accumulation cannot be attributed to a pleiotropic effect of chloroplast arrest, as it was not observed in seedlings treated with norflurazon (Fig. 9B). Norflurazon also causes a chloroplast arrest (Oelmüller, 1989), but should not directly affect the levels of IPP and DMAPP. Similarly to *Arabidopsis*, high accumulation of the DXS protein appears to result from a post-transcriptional regulatory mechanism, because the transcript levels of *dxs1*, *dxs2*, or *dxs3* genes displayed minimum variation during fosmydomicin treatment (Fig. 9C).

Discussion

The MEP pathway provides the structural molecules for a variety of key metabolites. Understanding the regulatory mechanisms of this biosynthetic route is important for the potential modulation of the production of key isoprenoids. Until now, such attempts to regulate exogenously the production of the diverse metabolites derived from this pathway have met with limited success. This is probably because changes in the levels of some primary metabolites synthesized from this pathway, such as hormones, result in pleitropic effects on plant development. This is particularly relevant since most enzymes of the MEP pathway are encoded by a single-copy gene in plants (Rodríguez-Concepción and Boronat, 2002; Cordoba *et al.*, 2009). An exception to this is DXS, represented in most plants by a small gene family (Cordoba *et al.*, 2009). Published work supports that the different genes that constitute the DXS family might be primarily responsible for the production of particular isoprenoids (Walter *et al.*, 2002; Phillips *et al.*, 2007; Floß *et al.*, 2008). In addition, diverse experimental data have demonstrated that DXS in plants limits output from the MEP pathway (Lois *et al.*, 2000; Estévez *et al.*, 2001; Enfissi *et al.*, 2005; Carretero-Paulet *et al.*, 2006). In consequence, understanding the molecular mechanisms that regulate the function and expression of the different isoforms and their roles in the synthesis of specific compounds is relevant. So far, the functionality of only a few members of the DXS family has been analysed in detail. The data obtained from these analyses show inconsistent scenarios. In *P. abies*, for example, it has been found that all members of the family encode functional proteins that participate differentially in the synthesis of particular isoprenoids (Phillips *et al.*, 2007). In contrast, in *Pinus densiflora* and in *Arabidopsis*, only some genes of the family appear to encode active proteins (Phillips *et al.*, 2008; Kim *et al.*, 2009). In the present work, the existence of three *dxs* genes in maize was demonstrated, each one encoding functional proteins with plastid localization. This is consistent with published data (Vallabhaneni and Wurtzel, 2009). The expression patterns of these genes and some of the regulatory mechanisms that modulate their expression at the transcriptional and post-transcriptional levels are defined.

The present sequence analyses of the *dxs* maize genes demonstrated that *dxs3* is the most divergent member of the family. In the maize DXS3 protein, several of the key amino acids that are proposed to be involved in the TPP and in the GAP-binding domains in bacteria are not conserved (Xiang *et al.*, 2007). Despite these changes, complementation analysis in *E. coli* demonstrated that this enzyme is active as a DXS, although with lower complementation efficiency than DXS1. However, the difference in complementation rates is not easily attributable to the amino acids changes present in DXS3. Complementation efficiency similar to DXS3 is observed with DXS2 even though this enzyme has most of these key amino acids present. A possible explanation of the lower complementation rates could be due to differences in the codon usage between maize and *E. coli*. Nonetheless, the complementation analysis clearly demonstrates that the DXS3 protein has DXS activity. Thus, the changes present in DXS3 are not essential for its enzymatic activity. The consequences, if any, of the changes present in

the DXS proteins from clade 3 remain to be analysed in the future, when reliable enzymatic assays from protein extracts are available. However, these differences open up interesting possibilities for enzymatic differences among these proteins and their functionality.

A recent publication linked the *dxs3* gene type specifically to the *Poaceae* family (Vallabhaneni and Wurtzel, 2009). However, the present phylogenetic analysis demonstrates that homologues to the *dxs3* genes are also present in dicots, such as *Populus trichocarpa*, *Vitis vinifera*, and *Arabidopsis*. Therefore, this gene type is conserved among plants. The emergence of this group occurred early in evolution prior to the monocot and dicot divergence. Thus, in addition to the two recognized phylogenetic DXS groups (Walter *et al.*, 2002; Krushkal *et al.*, 2003) evidence is provided for a third DXS group conserved among angiosperms. An exception to this is *Arabidopsis* where no *DXS* gene from clade 2 exists. Even though three independent *DXS* genes exist, two of them, *DXS1*/*CLA1* and *DXS2*/*DXL1* (At3g21500), group into the same clade (DXS1). It remains be determined if the *DXS3*/*DXL2* gene from *Arabidopsis* that belongs to clade 3 is functional. Based on microarray data from the public Genevestigator database, this gene is expressed in stamens and pollen (Zimmermann *et al.*, 2004).

Differential expression of the dxs *genes in maize*

In the present report, marked differences in the expression pattern and transcript levels of the three *dxs* maize genes were observed. Similar to *Arabidopsis* and tomato (Mandel *et al.*, 1996; Lois *et al.*, 2000), the maize *dxs1* is highly expressed in photosynthetic tissues during early development. This expression is consistent with the idea that this gene is committed to primary metabolism in stages where the photosynthetic apparatus develops. However, *dxs1* is not expressed equally along the leaves but accumulates at higher levels in leaf sheaths, a more C_3-like tissue (Langlade *et al.*, 1995).

In contrast, the highest transcript accumulation of *dxs2* is found in roots, mature leaves, and yellow kernels. The present analysis also shows two different types of messengers that differ by ~20 amino acids at their N-termini and have differential expression in these tissues. These differences do not change the predicted mature DXS2 protein but do change their subcellular localization. It was demonstrated that only the protein encoded by transcript T1 is transported into chloroplasts, where it is expected to perform its function. Previous reports have proposed that proteins that belong to the DXS2 group are committed to secondary metabolism (Walter *et al.*, 2002; Paetzold *et al.*, 2010). This idea is entirely consistent with the present results since the chloroplast DXS2 protein derived from the T1 transcript accumulates in roots and ears but apparently not (or at very low levels) in mature leaves. The maize *dxs2* gene is preferentially expressed in young seedling roots. Based on the development of the root system in maize, it is assumed that at the stage used for this analysis, the majority of *dxs2* expression derives from the primary root. It is possible that the decrease in the *dxs2* transcript level observed at day 20 coincides with the decay in activity of the primary root (Feldman, 1993).

The function of the protein encoded by the *dxs2* transcript T2, if any, is at the moment unknown. So far no other protein of the MEP pathway has been identified in the cytoplasm. However, it is also clear that DXP is transported into the chloroplast as shown by the chemical complementation of the *cla1* mutant (Estévez *et al.*, 2000). It is important to remember that DXS is also needed for the synthesis of B1 and B6 vitamins, although an alternative pathway has been found for the biosynthesis of B6 (Tambasco-Studart *et al.*, 2005).

The present data show that the *dxs2* transcripts (T1 and T2) initiate ~600 bp apart. Thus, each of these transcripts is probably transcribed from independent promoters. One promoter (T2) is located within the T1 intron, while the other promoter (T1) is located upstream of the transcription initiation site of the T1 transcript. A similar mechanism has been documented in maize for the differential expression of the pyruvate orthophosphate dikinase (PPDK) chloroplast and cytosolic genes (Sheen, 1991). It is possible that the proper transcriptional factors required to initiate transcription of T1 are only present in roots or ears but not in tissues like leaves. The sequence that encodes the N-terminus of the *dxs2* transit peptide, which corresponds to the first exon of the T1 transcript from maize, is almost 100% identical to that in rice and *Sorghum* (Vallabhaneni and Wurtzel, 2009). In general the sequences of the chloroplast transit peptides are remarkably heterogenous (Jarvis, 2008). This extraordinary conservation at the nucleotide level suggests that this exon has been acquired recently during the monocotyledon evolution. The transcript of *dxs3* is detected in most of the tissues analysed, albeit at lower levels than the other two genes. Its highest accumulation is found in mature leaves, husks, and immature ears. In a previous publication the *dxs3* transcript was correlated with the carotenoid accumulation in kernels (Vallabhaneni and Wurtzel, 2009). This observation contrasts with the present analysis, where no major differences in the accumulation of the *dxs3* transcript are observed between yellow and white mature kernel varieties. To clarify these discrepancies it will be important to analyze the level of dxs2 T1 transcript without the interference of the *dxs2* T2 transcript in the different maize germplasm. The present data show that the yellow kernels accumulate high levels of the *dxs2* transcript and high levels of the DXS protein.

As mentioned, the *dxs3* gene is expressed at low levels, in the tissues analysed. It is possible that this gene could participate in the synthesis of some products derived from this pathway such as hormones (gibberellic acid and abscisic acid), that are essential for plant survival but which are required at much lower levels compared with other isoprenoids derived from the same pathway (Lichtenthaler, 1999; Rohmer, 1999; Kasahara *et al.*, 2002). Such a role would also explain why the expression of the *dxs3* gene is

apparently not modulated by light, since these hormones would be required in a variety of tissues.

Finally, in this work the level of the DXS protein in maize was also analysed using heterologous antibodies raised against *Arabidopsis* DXS1. DXS is a protein highly conserved in evolution and the antibodies used here reproducibly detect a discrete band with the expected molecular mass for the DXS protein in various maize tissues. Similar to other plants, maize DXS accumulates at high levels in green photosynthetic tissues. High levels of this protein were also detected in yellow kernels. If it is assumed that protein abundance reflects transcript levels, then the proteins detected most probably correspond to *dxs1* in young leaves and *dxs2* in the yellow kernels. This accumulation correlates with a higher demand for IPP and DMAPP in these tissues. These results support that in maize, DXS also has a limiting role in this pathway, as in other plants (Estévez *et al.*, 2001; Carretero-Paulet *et al.*, 2006).

Previous reports showed that the level of the DXS protein in *Arabidopsis* is post-transcriptionally regulated by a feedback mechanism that apparently depends on the pool of IPP and DMAPP products (Guevara-García *et al.*, 2005; Rodríguez-Villalón *et al.*, 2009). The results presented in this work demonstrate that in maize the level of the DXS protein also accumulates in seedlings grown in the presence of the MEP pathway inhibitor fosmidomycin (Kuzuyama *et al.*, 1998; Zeidler *et al.*, 1998). The results support that the pathway flow also regulates the accumulation of DXS in maize through a post-transcriptional mechanism. Thus, this regulation appears to be an evolutionarily conserved mechanism and supports the fundamental role that DXS regulation has in this pathway. Similar to *Arabidopsis*, the accumulation of the DXS enzyme by fosmidomycin treatment results in a feedback mechanism in response to the low levels of some products derived from this pathway. As the DXS accumulation is not observed with norflurazon treatment, which affects carotenoid biosynthesis, it can be excluded that these compounds or the hormones derived from this pathway are involved in this regulation.

In conclusion, this study reports a detailed analysis of the expression profile of three *dxs* genes during maize development. Some of the regulatory mechanisms that modulate the expression levels of both genes and protein in response to internal and external signals are also demonstrated. This work extends previous analyses of these genes, and begins to uncover the complexity of the regulatory mechanisms that modulate this key enzyme for the MEP pathway in maize.

Acknowledgements

We are grateful to Dr Virginia Walbot and Dr. Mari Salmi for their helpful comments and Dr Axel Tiessen for providing us with some maize tissues. We thank Andrés Saralegui for his help with the confocal microscopy. We gratefully acknowledge the maize genomics database for providing maize lines and the EST clones used in this work. This work was supported by grants from CONACYT (40501-Q), DGAPA IN204503-3, BASF, and the Howard Hughes Medical Institution to PL and BIO200-00432 and CSD2007-00036 to MRC. Sequence data from this article have been deposited in GenBank.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–33402.

Bouvier F, d'Harlingue A, Suire C, Backhaus RA, Camara B. 1998. Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. *Plant Physiology* **117,** 1423–1431.

Campos N, Rodríguez-Concepción M, Sauret-Gueto S, Gallego F, Lois LM, Boronat A. 2001. *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate: a novel system for the genetic analysis of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis. *Biochemical Journal* **353,** 59–67.

Carretero-Paulet L, Cairó A, Botella-Pavía P, Besumbes O, Campos N, Boronat A, Rodríguez-Concepción M. 2006. Enhanced flux through the methylerythritol 4-phosphate pathway in *Arabidopsis* plants overexpressing deoxyxylulose 5-phosphate reductoisomerase. *Plant Molecular Biology* **62,** 683–695.

Chahed K, Oudin A, Guivarc'h N, Hamdi S, Chénieux J-C, Rideau R, Clastre M. 2000. 1-Deoxy-d-xylulose 5-phosphate synthase from periwinkle: cDNA identification and induced gene expression in terpenoid indole alkaloid-producing cells. *Plant Physiology and Biochemistry* **38,** 559–566.

Church GM, Gilbert W. 1984. Genomic sequencing. *Proceedings of the National Academy of Sciences, USA* **81,** 1991–1995.

Cordoba E, Salmi M, León P. 2009. Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. *Journal of Experimental Botany* **60,** 2933–2943.

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS. 2006. Gateway-compatible vectors for plant functional genomics and proteomics. *The Plant Journal* **45,** 616–629.

Eisenreich W, Bacher A, Arigoni D, Rohdich F. 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cellular and Molecular Life Sciences* **61,** 1401–1426.

Eisenreich W, Rohdich F, Bacher A. 2001. Deoxyxylulose phosphate pathway to terpenoids. *Trends in Plant Science* **6,** 78–84.

Emanuelsson O, Nielsen H, von Heijne G. 1999. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Science* **8,** 978–984.

Enfissi EMA, Fraser PD, Lois LM, Boronat A, Schuch W, Bramley PM. 2005. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnology Journal* **3,** 17–27.

Estévez JM, Cantero A, Reindl A, Reichler S, León P. 2001. 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *Journal of Biological Chemistry* **276,** 22901–22909.

Estévez JM, Cantero A, Romero C, Kawaide H, Jiménez LF, Kuzuyama T, Seto H, Kamiya Y, León P. 2000. Analysis of the expression of *CLA1*, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2- *C*-methyl-D-erythritol-4-phosphate pathway in Arabidopsis. *Plant Physiology* **124,** 95–103.

Feldman L. 1993. The maize root. In: Freeling M, Walbot V, eds. *The maize handbook*. New York: Springer-Verlag, 29–37.

Floß DS, Hause B, Lange PR, Küster H, Strack D, Walter MH. 2008. Knock-down of the MEP pathway isogene *1-deoxy-D-xylulose 5-phosphate synthase 2* inhibits formation of arbuscular mycorrhiza-induced apocarotenoids, and abolishes normal expression of mycorrhiza-specific plant marker genes. *The Plant Journal* **56,** 86–100.

Guevara-García AA, San Roman C, Arroyo A, Cortés ME, Gutiérrez-Nava ML, León P. 2005. The characterization of the Arabidopsis *clb6* mutant illustrates the importance of post-transcriptional regulation of the methyl-D-erythritol 4-phosphate pathway. *The Plant Cell* **17,** 628–643.

Hahn FM, Eubanks LM, Testa CA, Blagg BS, Baker JA, Poulter CD. 2001. 1-Deoxy-D-xylulose 5-phosphate synthase, the gene product of open reading frame (ORF) 2816 and ORF 2895 in *Rhodobacter capsulatus*. *Journal of Bacteriology* **183,** 1–11.

Harker M, Bramley PM. 1999. Expression of prokaryotic-1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. *FEBS Letters* **448,** 115–119.

Hsieh MH, Goodman HM. 2005. The Arabidopsis *IspH* homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis. *Plant Physiology* **138,** 641–653.

Jarvis P. 2008. Targeting of nucleus-encoded proteins to chloroplasts in plants. *New Phytologist* **179,** 257–285.

Kasahara H, Hanada A, Kuzuyama T, Takagi M, Kamiya J, Yamaguchi S. 2002. Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in *Arabidopsis*. *Journal of Biological Chemistry* **277,** 45188–45194.

Kim BR, Kim SU, Chang YJ. 2005. Differential expression of three 1-deoxy-D-xylulose-5-phosphate synthase genes in rice. *Biotechnology Letters* **27,** 997–1001.

Kim YB, Kim SM, Kang MK, Kuzuyama T, Lee JK, Park SC, Shin SC, Kim SU. 2009. Regulation of resin acid synthesis in *Pinus densiflora* by differential transcription of genes encoding multiple 1-deoxy-D-xylulose 5-phosphate synthase and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase genes. *Tree Physiology* **29,** 737–749.

Krushkal J, Pistilli M, Ferrell KM, Souret FF, Weathers PJ. 2003. Computational analysis of the evolution of the structure and function of 1-deoxy-D-xylulose-5-phosphate synthase, a key regulator of the mevalonate-independent pathway in plants. *Gene* **313,** 127–138.

Kuzuyama T, Takagi M, Kaneda K, Dairi T, Seto H. 1998. Direct formation of 2-C-methyl-D-erythritol-4-phosphate from 1-deoxy-D-xylulose 5-phosphate by 1-deoxy-D-xylulose 5-phosphate reductoisomerase, a new enzyme in the non-mevalonate pathway to isopentenyl diphosphate. *Tetrahedron Letters* **38,** 4509–4512.

Kuzuyama T, Takagi M, Takahashi S, Seto H. 2000. Cloning and characterization of 1-deoxy-D-xylulose 5-phosphate synthase from *Streptomyces* sp. strain CL190, which uses both the mevalonate and nonmevalonate pathways for isopentenyl diphosphate biosynthesis. *Journal of Bacteriology* **182,** 891–897.

Lange BM, Croteau R. 1999. Genetic engineering of essential oil production in mint. *Current Opinion in Plant Biology* **2,** 139–144.

Lange BM, Rujan T, Martin W, Croteau R. 2000. Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proceedings of the National Academy of Sciences, USA* **97,** 13172–13177.

Lange BM, Wildung MR, McCaskill D, Croteau R. 1998. A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. *Proceedings of the National Academy of Sciences, USA* **95,** 2100–2104.

Langlade JA, Hall LN, Roth R. 1995. Control of cellular differentiation in maize leaves. *Philosophical Transactions of the Royal Society B: Biological Sciences* **350,** 53–57.

Laule O, Fürholz A, Chang H, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange M. 2003. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in. *Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA* **100,** 6866–6871.

Lichtenthaler HK. 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 47–65.

Logemann J, Schell J, Willmitzer L. 1987. Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163,** 16–20.

Lois LM, Campos N, Rosa-Putra S, Danielsen K, Rohmer M, Boronat A. 1998. Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. *Proceedings of the National Academy of Sciences, USA* **95,** 2105–2110.

Lois LM, Rodríguez-Concepción M, Gallego F, Campos N, Boronat A. 2000. Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. *The Plant Journal* **22,** 503–513.

Mandel MA, Feldmann KA, Herrera-Estrella L, Rocha-Sosa M, León P. 1996. *CLA1*, a novel gene required for chloroplast development, is highly conserved in evolution. *The Plant Journal* **9,** 649–658.

Morris WL, Ducreux LJ, Hedden P, Millam S, Taylor MA. 2006. Overexpression of a bacterial 1-deoxy-D-xylulose 5-phosphate synthase gene in potato tubers perturbs the isoprenoid metabolic network: implications for the control of the tuber life cycle. *Journal of Experimental Botany* **57,** 3007–3018.

Nelson T, Langdale JA. 1989. Patterns of leaf development in C4 plants. *The Plant Cell* **1,** 3–13.

Oelmüller R. 1989. Photooxidative destruction of chloroplasts and its effect on nuclear gene expression and extraplastidic enzyme levels. *Photochemistry and Photobiology* **49,** 229–239.

Okada A, Shimizu T, Okada K, Kuzuyama T, Koga J, Shibuya N, Nojiri H, Yamane H. 2007. Elicitor induced activation of the methylerythritol phosphate pathway toward phytoalexins biosynthesis in rice. *Plant Molecular Biology* **65,** 177–187.

Paetzold H, Garms S, Bartram S, Wieczorek J, Urós-Gracia EM, Rodríguez-Concepción M, Boland W, Strack D, Hause B, Walter MH. 2010. The isogene 1-deoxy-D-xylulose 5-phosphate synthase 2 controls isoprenoid profiles, precursor pathway allocation, and density of tomato trichomes. *Molecular Plant* **3,** 904–916.

Page RDM. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12,** 357–358.

Phillips MA, León P, Boronat A, Rodríguez-Concepción M. 2008. The plastidial MEP pathway: unified nomenclature and resources. *Trends in Plant Science* **13,** 619–623.

Phillips MA, Walter MH, Ralph SG, *et al*. 2007. Functional identification and differential expression of 1-deoxy-D-xylulose 5-phosphate synthase in induced terpenoid resin formation of Norway spruce (*Picea abies*). *Plant Molecular Biology* **65,** 243–257.

Rodríguez-Concepción M. 2004. The MEP pathway: a new target for the development of herbicides, antibiotics and antimalarial drugs. *Current Pharmaceutical Design* **10,** 2391–2400.

Rodríguez-Concepción M, Boronat A. 2002. Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiology* **130,** 1079–1089.

Rodríguez-Concepción M, Forés O, Martínez-García JF, González V, Phillips MA, Ferrer A, Boronat A. 2004. Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during Arabidopsis seedling development. *The Plant Cell* **16,** 144–156.

Rodríguez-Villalón A, Gas E, Rodríguez-Concepción M. 2009. Phytoene synthase activity controls the biosynthesis of carotenoids and the supply of their metabolic precursors in dark-grown Arabidopsis seedlings. *The Plant Journal* **60,** 424–435.

Rohdich F, Bacher A, Eisenreich W. 2005. Isoprenoid biosynthetic pathways as anti-infective drug targets. *Biochemical Society Transactions* **33,** 785–791.

Rohmer M. 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Natural Products Reports* **16,** 565–574.

Rohmer M, Seemann M, Horbach S, Bringer-Meyer S, Sahm H. 1996. Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. *Journal of the American Chemical Society* **118,** 2564–2566.

Römer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM. 2000. Elevation of the provitamin A content of transgenic tomato plants. *Nature Biotechnology* **18,** 666–669.

Sauret-Güeto S, Botella-Pavía P, Flores-Pérez U, Martínez-García JF, San Román C, León P, Boronat A, Rodríguez-Concepción M. 2006*a*. Plastid cues posttranscriptionally regulate the accumulation of key enzymes of the methylerythritol phosphate pathway in Arabidopsis. *Plant Physiology* **141,** 75–84.

Sauret-Güeto S, Urós EM, Ibañez E, Boronat A, Rodríguez-Concepción M. 2006*b*. A mutant pyruvate dehydrogenase E1 subunit allows survival of *Escherichia coli* strains defective in 1-deoxy-D-xylulose 5-phosphate synthase. *FEBS Letters* **580,** 736–740.

Schwender J, Müller C, Zeidler J, Lichtenthaler HK. 1999. Cloning and heterologous expression of a cDNA encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase of *Arabidopsis thaliana*. *FEBS Letters* **455,** 140–144.

Sheen J. 1991. Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. *The Plant Cell* **3,** 225–245.

Small I, Peeters N, Legeai F, Lurin C. 2004. Predotar: a tool for rapidly screening proteomes for N-terminal targeting sequences. *Proteomics* **4,** 1581–1590.

Sprenger GA, Schörken U, Wiegert T, Grolle S, De Graaf AA, Taylor SV, Begley TP, Bringer-Meyer S, Sahm H. 1997. Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isoprenoids, thiamin and pyridoxol. *Proceedings of the National Academy of Sciences, USA* **94,** 12857–12862.

Tambasco-Studart M, Titiz O, Raschle T, Forster G, Amrhein N, Fitzpatrick TB. 2005. Vitamin B_6 biosynthesis in higher plants. *Proceedings of the National Academy of Sciences, USA* **102,** 13687–13692.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24,** 1596–1599.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Vallabhaneni R, Wurtzel ET. 2009. Timing and biosynthetic potential for carotenoid accumulation in genetically diverse germplasm of maize. *Plant Physiology* **150,** 562–572.

Walter M, Fester T, Strack D. 2000. Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the yellow pigment and the other apocarotenoids. *The Plant Journal* **21,** 571–578.

Walter M, Hans J, Strack D. 2002. Two distantly-related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. *The Plant Journal* **31,** 243–254.

Xiang S, Usunow G, Lange G, Busch M, Tong L. 2007. Crystal structure of 1-deoxy-D-xylulose 5-phosphate synthase, a crucial

enzyme for isoprenoids biosynthesis. *Journal of Biological Chemistry* **282,** 2676–2682.

Zeidler J, Schwender J, Müller C, Wiesner J, Weidemeyer C, Beck E, Jomaa H, Lichtenthaler K. 1998. Inhibition of the non-mevalonate 1-deoxy-D-xylulose-5-phosphate pathway of plant isoprenoid biosynthesis by fosmidomycin. *Zeitscrift für Naturforschung* **53c,** 980–986.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiology* **136,** 2621–2632.